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TITLE: SEQUENCE-BASED DIAGNOSIS**TECHNICAL AREA OF THE INVENTION**

5 The present invention relates to the area of cancer diagnostics. More particularly, the invention relates to the detection of alteration in cancer-related genes derived from a neoplasia sample and the use thereof for prognostic purposes.

BACKGROUND OF THE INVENTION

10 Breast cancer is the most common cancer in women. Although it is recognized that breast cancer tends to run in families, unpredictable acquired somatic mutations are responsible for the large majority of cases. There is today an underlying controversy in the prediction of outcome when
15 a woman is diagnosed as having breast cancer. Thus, when a lesion is discovered in a woman's breast, the diagnosis, cancer or not, is carried out on the basis of morphological change of the tumour and surrounding tissue. However, the prognosis or outcome influences the clinician's choice of
20 treatment considerably. Prognostic factors can be divided into two categories, i.e. biological and chronological factors.

The determination of biological factors include cytological examination of a needle biopsy of the tumour.
25 Immunohistochemical staining is used to investigate the presence and quantity of hormone receptors, and DNA labelling methods quantify the amount of DNA in the cells and DNA synthesis. Chronological factors include tumour size and axillary nodal status, the latter being the
30 traditional prognostic factor in the management of breast cancer.

In case cancer is diagnosed, the 20-30 lymph nodes are removed surgically, and the number of nodes containing cancer cells are counted. If more than a finite number of
35 nodes (e.g. five) is identified, the patient is exposed to radical treatment, surgically as well as radiation/chemotherapy or both. While the biological factors are being increasingly used to make treatment

decisions of the disease, lymph node status remains the standard against which the predictive power of biological prognostic factors are evaluated.

5 It is believed that patients with breast cancer that have axillary lymph node involvement have a relatively poor prognosis, partly due to other biological factors effecting aggressiveness and/or metastatic potential of the tumour, irrespective of the chronological stage at which the tumour is investigated. Recent findings suggest, however, that the
10 presence and extent of lymph node metastasis has little to do with tumour aggressiveness or metastatic potential, but is entirely a reflection of the relatively advanced chronological age of the tumor.

15 In the absence of more relevant prognostic factors of the outcome of the disease, most clinicians still rely on the clinical or chronological factors together with the morphological grade of the tumour in the microscope, factors which by the way have been used over 40 years.

20 There is therefore a need for more accurate indication of the biological status in the form of aggressiveness and metastatic potential available at early diagnosis which would enable the clinician to take steps to treat the patient earlier and more accurately. Thus, a small tumour with metastatic potential could be treated with radical
25 methods already from the initial diagnosis instead of waiting for relapse. Moreover, patients who do not have a tumour with metastatic potential where the risk for relapse is very small or non-existent, could be treated with milder methods.

30 During the last few years, research efforts have been directed to the finding of correlations between genetic mutation and cancer development and progression. An interesting type of genes in this context are the tumour suppressor genes, which are defined as genes for which
35 loss-of-function mutations are oncogenic. Wild-type alleles of such genes may function to prevent or suppress oncogenesis. An example of such a gene is the p53 gene on chromosome 17p which encodes the suppressor protein p53.

Mutations in the p53 gene can be found in about half the cases of human cancer. Cancer forms which have been found to have a strong correlation with mutations in the p53 gene are, for example, breast cancer and colon cancer. A method
5 of diagnosing human neoplasia or cancer, such as breast, colorectal or lung cancer, by detecting loss of wild-type p53 genes in a sample suspected of being neoplastic is disclosed in EP-A-390 323.

The kinds of mutations that make the tumour suppressor
10 genes defective vary between different tumour suppressor genes. Thus, whereas the tumour suppressor genes which are defective in e.g. retinoblastoma are commonly inactivated by nonsense mutations that cause truncation and instability of the protein, more than 90% of the mutations in p53 are
15 missense mutations that change the identity of an amino acid. Such amino acid changes can alter the conformation and thereby the stability of the p53 protein and can indirectly alter the sequence-specific DNA binding and transcription factor activity of the p53.

Recent results show that p53 plays an important role
20 in the control of DNA repair mechanisms, preventing DNA replication prior to cell division until repair is completed. It has also been found that there are hot-spots in the gene that are more prone to mutation, but the
25 mutations are in general acquired randomly and spontaneously within the hot-spot regions.

As far as breast cancer is concerned, a correlation has been observed between survival and p53 mutation. Thus, Thorlacius et al., Cancer Res. 53 (1993) 1637-1641 report
30 that women with a p53 mutation in the breast tumour run a more than threefold higher risk of dying than those without a p53 mutation.

Apart from the above correlation with survival, however, analysis of p53 mutations in breast tumours as
35 well as in other tumours has failed to establish any correlation with clinical parameters and prognosis in other respects.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for diagnosing, based on the detection of possible mutations in a cancer-related gene, such as the p53 gene, from neoplastic tissue, blood or other body fluid with regard to biological status in the form of aggressiveness and metastatic potential to enable an accurate prediction of the disease and thereby an adequate treatment, especially at early diagnosis.

10 In accordance with the invention, it has now been found that by determining from a human neoplasia sample from an afflicted patient the location and nature of a mutation in a cancer-related gene, the severity of the detected changes for the outcome of the patient may be
15 evaluated. Of particular relevance is the detection of mutations in those parts of the gene which encode a biologically functional domain or domains of the protein.

The invention therefore provides a method of diagnosing a human neoplasia in a tissue, blood or other
20 body fluid sample (e.g. urine, sputum), which comprises analysing from genomic DNA or cDNA derived from said neoplasia the DNA sequence of a gene encoding a cancer-related protein for the presence of mutations therein, determining from the presence, nature and location of any
25 such mutation or mutations the influence thereof on the biological function of the corresponding protein and thereby on the properties of the neoplasia, and on the basis thereof prognosticating the development of the neoplasia.

30 The expression "cancer-related gene" as used herein means any gene for which a mutation may be correlated with the development of neoplasia or cancer. Such genes generally encode proteins taking part in the DNA replication cycle, such as suppressor proteins, oncogens
35 including growth inducing proteins, and regulatory proteins. Exemplary of such genes are, besides the p53 gene already mentioned above, those encoding the proteins WAF1, erb B-2, HerII/Neu, p16, MTS I & II, MLH 1 & 2 and Ras.

The mutations to be detected include point mutations and deletions as well as polymorphisms.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of p53 cDNA with aligned coding region as well as four amplified and sequenced overlapping fragments thereof used in Example 1 below. On the fragments 1 to 4, primers are indicated by "←". "B" indicates a biotinylated primer and "S" indicates a sequencing primer.

Fig. 2 is a representation of the DNA sequences of the primers schematically indicated in Fig. 1. Also the position in the p53 gene in relation to A in the ATG start codon is indicated.

DETAILED DESCRIPTION OF THE INVENTION

The p53 protein structure as well as various mutations detected therein has been described inter alia by Harris, C., Science 262 (1993) 1980-1981. As shown therein, p53 has a transactivation domain, an oligomerization domain, and four evolutionary conserved regions. The complete DNA sequence of the normal or wild type p53 gene may be found in, for example, Zakut-Houri, R., et al., EMBO J. 4 (1985) 1251-1255, GenBank, entry HUM53C (cDNA sequence), as well as in Mol. Biol. Cell. 6 (1986) 1379-1385 and Mol. Cell. Biol. 7 (1987) 961-963, LMBL database, entry HSP53G (genomic DNA sequence).

In accordance with the invention, it has now been found that, especially with respect to breast cancer, there is a relationship between (i) the position of the mutation in p53 cDNA, (ii) the evolutionary conserved and functional region in the protein, and (iii) the amino acid transition.

It has also been found that a mutation or mutations in the p53 gene mediate a poor prognosis for the breast cancer patient, irrespective of other biological factors, like hormone receptor status or lymph node involvement at the initial presentation.

Further, on one hand, a tendency has been found that breast cancer patients who have not developed lymph node involvement have a mutation(s) in the p53 gene

predominantly located in the evolutionary conserved regions close to the DNA binding functional domain of the p53 protein. Such mutations mediate the a lower affinity binding to the specific motif or a non-specific binding to other regulatory motifs, thus effecting the expression of other genes in the DNA pathway.

On the other hand, a tendency has has been found that in breast cancer patients with lymph node metastases, the majority of p53 mutations are located in the conserved regions close to the transactivation site in the p53 protein. In several cases the mutation has given rise to a transcriptional stop signal which results in a truncated protein which lacks the transactivation site. This will "knock out" the protein in its role as a block in cell division while DNA proof-reading and repair takes place. The tumour cells will thereby be anachistic, resulting in a fast growing aggressive tumour.

Thus, by analysing the distribution of the mutations by DNA sequencing at least throughout the part or parts of the p53 gene which encode biologically functional domains, it is possible to distinguish between (i) mutations detrimental to the patient, e.g., those defined above affecting the DNA binding or transactivation, and (ii) mutations less harmful for the patient, i.e. amino acid changes not greatly effecting structure or function.

By determining the DNA sequence of the p53 gene in a malignant sample and classifying the mutations with respect to tumour aggressiveness and metastatic potential in accordance with the above, the clinician will be provided with a reliable prognostic factor correlating to the incidence of relapse. The treatment, in the form of minor or radical surgery, with or without radiation or chemotherapy, can then be designed accordingly. For example, patients lacking other alarming factors but with a p53 mutation in a critical region, who today would be subjected to milder treatment forms, could be treated with radical treatment already from the first diagnosis. Likewise, women with e.g. lymph node involvement but with a

non-critical p53 mutation, who today would receive radical treatment, could have a milder treatment. This would, of course, have an effect on both treatment costs and unnecessary suffering.

5 What has been said above about mutations in p53 and breast cancer is, of course, also applicable to neoplastic changes in other organs, such as lung, prostate, gastric and colorectal cancer as well as leukemia and malignant melanoma. Similarly, the inventive concept is applicable to
10 other cancer-related genes than the p53 gene as described above.

Methods for sample preparation and DNA sequencing and data interpretation are known per se in the art and will therefore not be particularly described herein. An
15 innovative method for the handling of multiple clinical samples for analysing a gene for mutations, which method, especially with respect to the p53 gene, is a separate aspect of the present invention, comprises the following steps:

- 20 (i) sample preparation,
(ii) amplification of genomic DNA or cDNA,
(iii) processing of the amplified product(s), preferably using a solid phase technique,
(iv) detection on an automated sequencer, and optionally
25 (v) use of computer software to track and control the sample and process steps and/or to aid in and/or interpret the sequence data obtained.

In the sample preparation step, either genomic DNA is prepared or cDNA is prepared from mRNA.

30 Amplification of the DNA is preferably performed by PCR, although other amplification techniques are, of course, also conceivable. In the case of PCR, one of the primers is preferably provided with a "separation handle", e.g. a biotinyl group.

35 In the solid phase processing of the amplified DNA, the DNA fragments are captured on a solid support, such as by binding of a biotinylated DNA fragment to a solid support with immobilized avidin or streptavidin. After

melting off the non-biotinylated DNA strand, the sequencing primers are annealed to the immobilized DNA fragments and sequencing reactions with the four dNTP's and respective terminators, such as ddNTP's, are performed with the
5 immobilized DNA fragments as templates, as is per se known in the art.

The primer extension products are then electrophoretically separated and detected on an automated nucleic acid sequencer.

10 Preferably, especially with respect to the p53 gene, several overlapping fragments are amplified and sequenced.

The solid support may be in bead form, such as magnetic beads. A preferred solid phase processing system is, however, disclosed in our WO 94/00597 and WO 94/11529
15 (the entire disclosures of which are incorporated by reference herein) and comprises a multi-pronged device, usually a comb-like element, the pin tips or teeth of which constitute the immobilization surfaces.

Computer software may be used on two levels, (i) for
20 tracking the different samples throughout the processing and analysis and controlling the different process steps, and (ii) for at least aiding in the interpretation of the sequence data obtained.

Hereinafter, the invention will be illustrated by the
25 following non-limiting example.

EXAMPLE

Tumour samples from 107 breast cancer patients with identified node status (node negative or node positive) were prepared and sequenced as follows.

30 Preparation of mRNA from patient sample

300 µl of RNazole™ (phenol and GTC, Cinna/Biotechx Lab Inc., Houston, Texas, U.S.A.) were added to a 1.5 ml tube and placed on ice. A 5 x 2 x 2 mm piece of frozen tissue sample was cut and ground in the extraction solution in the
35 tube using a micro pestle. 500 µl of RNazole™ and 80 µl of chloroform/isoamyl alcohol (24:1) were then added, vortexed for 10 secs and left on ice for 5 min. After centrifugation for 10 mins, 350 µl of the upper phase was transferred to a

new tube containing 350 μ l isopropanol and mixed by vortex. The tube was then placed on ice for 30 min and centrifuged at maximum speed for 20 min. The resulting pellet was washed twice with 70% ethanol, dried briefly and dissolved
5 in 50 μ l of DEPC-treated water and 25 u (1 μ l) RNAGuard[®] (a nuclease inhibitor, Pharmacia Biotech AB, Uppsala, Sweden).

For each set of RNA isolations made, a negative control (no tissue added) was processed in the same way.

10 Preparation of cDNA

The RNA sample obtained above was heat denaturated at 90 °C for 3 min and quenched on ice. 37.5 μ l of 2 x cDNA mix (90 mM Tris-HCl, pH 8.3, 138 mM KCl, 18 mM MgCl₂, 30 mM DDT, 3.6 mM dATP, dCTP, dTTP, dITP and 0.9 mM dGTP, 0.152
15 UA₂₆₀U₂₆₀(N)₆), 10 μ l of MMULV reverse transcriptase (RT) (200 u) and 2.5 μ l of RNAGuard[®] (62.5 u) were mixed in a tube and 25 μ l of the denaturated RNA sample were added. After incubating for 1 h at 37 °C, the cDNA reaction was heat denaturated at 90 °C for 3 min, and the cDNA samples
20 were stored at -20 °C.

For each set of cDNA reactions made, a negative control (25 μ l of water instead of RNA sample) was processed in the same way.

PCR amplification of cDNA

25 Four different fragments of the cDNA (Fragments 1 to 4 in Fig. 1) were amplified in separate reactions, using the PCR primers shown in Fig. 2. Each reaction was performed in a Perkin Elmer 9600 PCR machine (Perkin Elmer-Cetus, Emeryville, California, U.S.A.) as follows:

30 In a 0.2 ml tube were mixed 5 μ l of PCR II buffer (10x) (Perkin Elmer-Cetus, Emeryville, California, U.S.A.), 5 μ l of 5'-primer (1 pmol/ μ l), 5 μ l of 3'-primer (1 pmol/ μ l), 1.2 μ l of 25 mM MgCl₂, 28 μ l of water and 0.8 μ l of AmpliTaq polymerase (4 u) (Perkin Elmer-Cetus, Emeryville,
35 California, U.S.A.). 5 μ l of cDNA sample or 5 μ l of negative control sample were added (total PCR reaction = 50 μ l). The samples were cycled 38x with the AUTO profile: 94 °C for 15 sec, 58 °C for 30 sec, 72 °C for 45 sec. The

amplification reaction was ended with a 5 min HOLD at 72 °C and linked to HOLD file 4 °C \Rightarrow x. Purity, quality and quantity were checked by running 5 μ l of the PCR reaction on a 1 % agarose gel with 0.2 μ g of the 100 Base-Pair Ladder (molecular weight marker, Pharmacia Biotech AB, Uppsala, Sweden) as reference.

DNA sequencing

Sequencing of the four DNA fragments obtained above were performed in an A.L.F.TM DNA Sequencer (Pharmacia Biotech AB, Uppsala, Sweden). The sequencing reactions were performed using comb-like polystyrene manifolds and corresponding well plates as described in our WO 94/11529. Each comb had 8 teeth, and the well plates were of two types, one type with wells designed to receive four comb teeth, below referred to as "four teeth well", and a second type with each well designed to receive a single comb tooth, below referred to as "one tooth well".

The following fragments of the p53 gene were sequenced, using the sequencing primers shown in Fig. 2:

20

	<u>Designation</u>	<u>Exons</u>	<u>Base pairs</u>
	SILS	2 to half 4	316 to 136
	SIL	2 to 5	575 to 136
	FF1	5 to 8	523 to 936
25	RF2	6 to 9	1080 to 739
	ESP	9 to 11	1060 to TGA (stop)

1. The PCR product obtained above (40 μ l) was transferred to a "four teeth well" containing 80 μ l of BW buffer (1 x TE, 2 M NaCl). Mixing was performed by pipetting, avoiding bubbles. The avidin-coated tips of a comb were inserted into the well and dipped a couple of times to improve the capture of biotinylated PCR product to the comb and were then left at room temperature for at least 60 min.
2. The comb was then moved to another "four teeth well" containing 100 μ l of 0.1 M NaOH and incubated for 5 min for elution of the unbound DNA strands. The comb was then

washed once in 100 µl of 0.1 NaOH, once in 100 µl of TE buffer and finally once in 100 µl of ultra-pure water.

3. To a new "four teeth well" were added 104 µl of water, 12 µl of 10 x Annealing buffer (AutoRead™ Sequencing Kit, Pharmacia Biotech AB, Uppsala, Sweden), 4 µl of a 1 pmol/µl fluorescein-labelled sequencing primer (see Fig. 2), and the comb was inserted into the well. The annealing mix was heated to 55 °C for 5 min and then left at room temperature for at least 10 min.

4. From previously prepared master mixes of Sequence-mix (2 µl 10x Annealing buffer, 1 µl Extension buffer (AutoRead™ Sequencing Kit, Pharmacia Biotech AB, Uppsala, Sweden), 4 µl d/ddNTP mix, 12 µl water, 1 µl (2 u) T7 polymerase diluted in Enzyme-dilution buffer (AutoRead™ Sequencing Kit, Pharmacia Biotech AB, Uppsala, Sweden)) for each d/ddNTP, stored on ice, with the T7 enzyme added as late as possible, 20 µl of each respective sequence-mix were dispensed in individual "one tooth wells". Immediately after that, the comb with annealed primer was inserted into the wells, incubated for 5 min at 37 °C and then placed on ice.

5. The loading wells of an A.L.F.™ DNA Sequencer gel prewarmed to 45 °C were rinsed and loaded with 15 µl Stop solution (AutoRead™ Sequencing Kit, Pharmacia Biotech AB, Uppsala, Sweden) to each well. The comb was removed from the "one tooth wells" above and inserted into the rinsed loading wells and left for 10 min to release the respectively terminated primer extension products. The comb was then carefully removed and the electrophoretic separation and detection process of the A.L.F.™ DNA Sequencer was started.

Results

15 samples were found to have mutations in the p53 gene. Details on the mutations are given in Table 1 (Node negative patients) and Table 2 (node positive patients) below. In the tables, "trans" is the region surrounding the transactivation site, "DNA" is the region surrounding the DNA binding domain, and "Bca" is breast cancer. "Amino

acid" refers to the position in the p53 protein. Under "Survival", "-" means death and "+" means survival.

Table 1

Node negative patients

5

	<u>Amino acid</u>	<u>Transition</u>	<u>Domain</u>	<u>Survival/ cause death</u>
	175	Arg→His	trans	- / BCa
	221	Glu→Asp	DNA	- / BCa
10	248	Arg→Trp	DNA	+
	248	Arg→Gln	DNA	- / BCa
	248	Arg→Gln	DNA	+

Table 2

Node positive patients

15

	<u>Amino acid</u>	<u>Transition</u>	<u>Domain</u>	<u>Survival/ cause death</u>
	36	Pro→Leu	trans	- / BCa
20	127	Ser→Phe	trans	- / BCa
	158	Arg→His	trans	- / BCa
	165	Gln→Lys	trans	+
	165	Gln→stop	trans	- / BCa
	165	Gln→stop	trans	- / BCa
25	193	His→Gln	trans	+
	248	Arg→Trp	DNA	+
	248	Arg→Gln	DNA	+
	273	Arg→His	DNA	- / BCa

30

As appears from the tables, both node positive and node negative patients with mutations in the conserved regions of p53 gene usually, depending, however, on the nature and location of the mutation, have a poor prognosis. The distribution of mutations shows that sequencing of at least large parts of the gene is necessary.

35

CLAIMS

1. A method for sequence-based diagnosis of a human neoplastic tissue, blood or other body fluid sample,
5 characterized by analysing from genomic DNA or cDNA derived from said neoplasia the DNA sequence of a gene encoding a cancer-related protein for the presence of mutations therein, determining from the presence, nature and location of any such mutation or mutations the influence thereof on
10 the biological function of the corresponding protein and thereby on the properties of the neoplasia, and on the basis thereof prognosticating the development of the neoplasia.
- 15 2. The method of claim 1, characterized in that said properties of the neoplasia includes biological aggressiveness and/or metastatic potential.
- 20 3. The method of claim 1 or 2, characterized in that said cancer-related protein is a protein taking part in the DNA replication cycle.
- 25 4. The method of claim 3, characterized in that the protein is a suppressor protein or a growth inducing protein.
- 30 5. The method of any one of claims 1 to 4, characterized by analyzing a part or parts of the gene which encode at least one biologically functional domain of the cancer-related protein.
- 35 6. The method of claim 5, characterized in that said biologically functional domain includes a DNA binding domain and/or transactivation site.
7. The method of claim 5 or 6, characterized in that evolutionary conserved regions of the gene are analyzed.

8. The method of claim 5 or 6, characterized in that the gene analyzed for mutations is selected from genes encoding the proteins p53, WAF1, erb B-2, HerII/Neu, p16, MTS I & II, MLH 1 & 2 and Ras.

5

9. The method of claim 8, characterized in that the gene encodes the p53 protein.

10. The method of claim 9, characterized in that the neoplasia is a breast, lung, prostate, gastric, colorectal, melanoma or leukemia neoplasia.

11. The method of claim 10, characterized in that said sample originates from a breast neoplasia.

15

12. The method of any one of claims 1 to 11, characterized in that it comprises one or more of the following steps: preparation of genomic DNA or cDNA, amplification of at least part of the cancer-related gene, processing of the cancer-related gene including sequencing reactions, and detection of the products from the sequencing reactions in an automated nucleic acid sequencer, computer software optionally being used to (i) track samples and control process steps and/or (ii) to aid in and/or interpret sequence data obtained.

25

13. A method of detecting mutations in a gene, characterized by comprising the steps of preparing genomic DNA or cDNA, amplifying at least part of the gene, processing the amplified DNA to produce sequencing reaction products, preferably by solid phase based techniques, detecting the sequencing reaction products in an automated nucleic acid sequencer to determine a DNA sequence or sequences of the p53 gene, and comparing the sequence or sequences with the corresponding wild type p53 gene sequence or sequences, computer software being used to (i) track samples and control process steps and/or (ii) to at least aid in interpreting sequence data obtained.

30
35

14. The method according to claim 13, characterized in that mutations are detected in a gene encoding the p53 protein.

ABSTRACT

A method for sequence-based diagnosis of a human neoplastic tissue, blood or other body fluid sample, comprises analysing from genomic DNA or cDNA derived from said neoplasia the DNA sequence of a gene encoding a cancer-related protein for the presence of mutations therein, determining from the presence, nature and location of any such mutation or mutations the influence thereof on the biological function of the corresponding protein and thereby on the properties of the neoplasia, and on the basis thereof prognosticating the development of the neoplasia.

Fragment 1

Position in p53
in relation to A
in start codon ATG

PCR primers

<i>PB1-22</i>	B-5'-GAC ACG CTT CCC TGG ATT GGC-3'	-88 / -28
<i>PT1-23</i>	S'-GCA AAA CAT CTT GTT GAG GGC A-3'	404 / 383

Sequence primer

<i>PF1-20</i>	F-5'-CAG GGG AGTACG TGC AAG TCA CAG-3'	497 / 385
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Fragment 2

PCR primers

<i>PB2-23</i>	B-5'-GTT TCC GTC TGG GCT TCT TGC A-3'	322 / 439
<i>PNT2-22</i>	S'-GGT ACA GTC AGA GCC AAC CTC-3'	689 / 669

Sequence primer

<i>PF2-24</i>	F-5'-GCC AAC CTC AGG CGG CTC ATA-3'	677 / 657
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Fragment 3

PCR primers

<i>PT3-1</i>	S'-TGG CCC CTC CTC AGC ATC TTA-3'	562 / 582
<i>BK1</i>	B-5'-CAA GGC CTC ATT CAG CTC TC-3'	1043 / 1024

Sequence primer

<i>PF3-6</i>	F-5'-CGA GTG GAA GGA AAT TTG CGT-3'	585 / 605
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Fragment 4

PCR primers

<i>PT4-3</i>	S'-CGG CGC ACA GAG GAA GAG AAT C-3'	843 / 864
<i>PB4-8</i>	B-5'-CGC ACA CCT ATT GCA AGC AAG GG-3'	1287 / 1264

Sequence primer

<i>PF4-10</i>	F-5'-GGG GAG CCT CAC CAC GAG CTG-3'	876 / 896
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B - Biotin

F - Fluorescein

FIG. 2

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